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Microbial *N*-Dealkylation of Atrazine: Effect of Exogeneous Organic Substrates and Behaviour of the Soil Microflora

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Abstract: It has been demonstrated that atrazine side-chain mineralisation could be substantially stimulated by addition of carbon-containing substrates such as cellulose, green manure, straw or sawdust in the presence of NH_4^+ nitrogen but poorly affected by amendments with glucose. Cellulose has the most beneficial effect. For that substrate it has been shown that (i) simultaneous application of the organic amendment and atrazine results in kinetics for side-chain dealkylation showing a lag phase which is reduced or even eliminated by preliminary incubation with the amendment, (ii) rate and extent of mineralisation of the ethylamino side chain are significantly accelerated by decreasing the C/N ratio of the amendment. By comparison, mineralisation of the isopropylamino side chain is not appreciably affected by a change in the value of the C/N ratio as far as atrazine is applied within a two- to three-week period following the organic treatment after which a small deficit in N supply has a definite beneficial effect on mineralisation.

Cellulose and, to a lesser extent, straw induce a biphasic change in bacterial number with more numerous and/or active atrazine degraders being predominantly found in the later-developing bacterial community. The fungal microflora is relatively unaffected by all types of carbon substrates but glucose and, unexpectedly, by atrazine at high ratio of application. Activation of atrazine mineralisation seems to be a co-metabolic process which is kinetically controlled by the rate of release from polymerised C substrates of easily available and readily metabolisable low-molecular-weight co-substrates. Transient production of glucose as an end-product of cellulose depolymerisation might induce catabolic repression of dealkylation enzyme systems and be responsible for a lag in atrazine side-chain mineralisation. © 1998 Society of Chemical Industry

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Key words: atrazine; *N*-dealkylation; organic amendments; cellulose; C/N ratio; biomass; bacterial counts; fungal counts

1 INTRODUCTION

Since their first application for agricultural weed control in corn, triazine herbicides have been recognised as persistent chemicals, with methoxytriazines being more persistent than chloro- and methylthiotriazines.[1] In normal circumstances they exhibit good weed control, but with a potential hazard of injury to the succeeding crop in the rotation.[2] Since the demonstration of a possible enrichment in previously treated soil,[3] there have been no documented instances of enhanced degradation in soil. Despite numerous reports on the potential for micro-organisms to transform these herbicides,[4–7] serious questions have been raised on the practical significance of microbial degradation of triazines in soils.[1] More recent researches have substantiated the

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role of micro-organisms in soil detoxification of *s*-triazine herbicides,[8] possibly in combination with abiotic hydrolytic processes.[9,10] More recently, complete mineralisation of atrazine by microbial consortia[11,12] or pure bacterial isolates[13,14] has been demonstrated. Accelerated degradation has also been shown in soils with a long history of maize cultivation which have received atrazine every year.[15] It is commonly accepted that, due to limited bioavailability, disappearance rates in soils are less than in pure culture.[12] Because of its persistence and its high leaching potential and also because it has been heavily used throughout the world, atrazine and its dechlorinated and dealkylated metabolites have become serious contaminants of rivers,[16,17] drainage waters[18–20] and groundwaters in the US,[21,22] in Australia,[23] in the Republic of South Africa[24] and in many European countries, including the UK,[25] Germany,[26] the Netherlands[27] and France.[28] For this reason use of this herbicide has been banned or restricted in many western countries.

In soils where prolonged persistence is observed, prevention of further increase in soil and water contamination could be tentatively obtained either after complementing the natural degrading potential by introduction of microbial species with increased degrading capacities or after stimulating the overall metabolic activity of the autochthonous microflora by addition of exogenous organic amendments. Contradictory results have been reported in the literature on the effects of organic amendments on pesticide degradation. Inhibition has been observed with sewage sludges,[29] but ample demonstration has been made of the beneficial effects of easily mineralisable organic material on pesticide degradation, especially if it is a co-metabolic process.[30] Reintroducing crop residues could offer a cheap and efficient way of achieving the necessary stimulation of the soil microflora to increase the degradation of pesticides.

The aim of the present work was to test the influence of different carbon sources, as chemically defined compounds such as glucose and cellulose, or as crop residues such as green manure and straw, at different rates of application and at different C/N ratios, on the transformation of atrazine in one soil with particular emphasis on side-chain dealkylation. Attempts were made to relate the observed modifications in the kinetics of side-chain mineralisation to changes in the size of the bacterial and fungal soil floras.

2 EXPERIMENTAL METHODS

2.1 The soil

The soil used had no history of atrazine treatments. It was sampled from the 0–15 cm top layer of an eutric cambisoil. The microbial biomass as well as the main physicochemical characteristics are given in Table 1.

TABLE 1
Main Physical, Chemical Characteristics and Biomass of the Soil Used

Sand (%)	*Silt* (%)	*Clay* (%)	*O.C* (%)	*N* (%)	*pH* (*water*)	*Biomass* (*mg C kg*$^{-1}$ *soil*)
3·7	57·7	38·6	1·33	0·16	7·3	191

Before use, the soil was partially air-dried to an appropriate water content (12–14% on a dry weight basis) and sieved. Only the fraction of soil aggregates between 2 and 3 mm was used. Pre-conditioned soil samples of soil aggregates (49 g on a dry weight basis) were humidified with deionised water. The amount of water was calculated to reach 80% of the soil water holding capacity (WHC), taking into account the volume of solutions of carbon substrates and pesticides.

2.2 Chemicals, organic compounds, culture media and soil treatments

Analytical grade non-labelled atrazine (99% purity, Riedel-de Haën) was used for preparing herbicide stock solutions. Ethyl- and isopropyl-chain-labelled [^{14}C]atrazine (specific activities of respectively 82·5 MBq mmol^{-1} and 33·7 MBq mmol^{-1} and radiochemical purity greater than 95%; Amersham) were used. Because of its poor solubility in water, ethanolic solutions of atrazine were prepared by dissolving the radiolabelled chemical and adjusting the final concentration with non-labelled chemical. These ethanolic solutions (100 µl) were added to small portions (1 g) of sieved soil (250 µm) which, after evaporation of the solvent by overnight gentle air-streaming, were introduced into the 49 g sample of soil.

Chemical grade non-labelled D-glucose (Rectapur™, Prolabo), cellulose for TLC (Whatman) as well as finely ground Italian rye-grass, wheat straw or poplar sawdust were used for enriching the soil. Preliminary determination of the water, C and N contents of the 'natural' substrates (Table 2) was necessary in order to adjust the amount of C introduced and the C/N ratios (Table 3). Each substrate was added as powder to each soil sample. The mixture was homogenised by shaking and the C/N ratio of the organic amendment was

TABLE 2
Composition of the Different Plant Residues

	Grass	*Straw*	*Sawdust*
Water content (%)	7·40	7·10	42
Organic matter content (%)	82·50	89·10	56·80
Nitrogen content (%)	3·20	0·47	0·12

TABLE 3
Amounts of the Different Organic Substrates and Corresponding C and N Contents[a]

	Grass	*Straw*	*Sawdust*	*Glucose*	*Cellulose*
Fresh matter	0·61	0·56	0·88		
Dry matter	0·56	0·52	0·51	0·50	0·50
O. C. content	0·25	0·25	0·25	0·20	0·22
N content	0·018	0·002	$6{\cdot}10^{-4}$	0	0
$(NH_4)_2SO_4$ supplied	0·033	0·108	0·113	0·094	0·104

[a] All quantities in g (100 g soil)$^{-1}$.

adjusted by addition of a solution of ammonium sulfate. No correction was made for N content of the soil, which was considered of no significance.

Bacteria and actinomycetes were counted on solid YPGA medium which had the following composition: glucose (10 g litre^{-1}, Prolabo), peptone (5 g litre^{-1}, Difco), yeast extract (5 g litre^{-1}, Difco), agar (15 g litre^{-1}, Difco). Cycloheximide (50 mg litre^{-1}, Sigma) was added as fungicide. Fungi were counted on solid malt extract medium with the following composition: malt extract (10 g litre^{-1}, Difco), agar (15 g litre^{-1}, Difco) and citric acid (25 ml litre^{-1} of a 10 g litre^{-1} solution, Prolabo), as bactericide.

2.3 First experiment

This was designed to compare the relative effects of glucose and cellulose, at the same rate of carbon enrichment and for two C/N ratios. The relative timing between the organic amendment and the chemical treatment was also tested. Two groups of samples of soil aggregates (49 g) received either glucose (2 g C kg^{-1} soil) or cellulose (2·2 g C kg^{-1} soil) plus the required volume of a solution of ammonium sulfate to adjust the C/N ratio to 10. Two other groups of soil samples were also treated with glucose or cellulose, but the C/N ratio was adjusted to 50. One last group of control samples was left unamended. The final water content of the soil was 80% of the WHC. All samples were then treated with [^{14}C]atrazine (3·33 mg kg^{-1}) as previously described. Addition of atrazine was made after different periods of preliminary incubation with the organic substrates: 0, 17, 39 and 75 days. Additionally, kinetics of mineralisation of [*ethyl*-^{14}C]atrazine (6·67 kBq per sample) and [*isopropyl*-^{14}C]atrazine (3·7 kBq per sample) were compared. Four replicates were prepared for each combination of treatments. Each soil sample was placed in a 250-ml glass jar and incubated before and after herbicide application in a closed 1-litre biometer flask containing two polyethylene scintillation vials, one with water (10 ml) for maintaining water saturation, the other with sodium hydroxide (0·2 M; 5 ml) to trap [^{14}C]carbon dioxide evolved from mineralisation of labelled atrazine. Samples were incubated in the dark in a climatic chamber (20°C ± 0·5°C) and were periodically aerated when changing the carbon dioxide traps for analysis of the radioactivity content of the sodium hydroxide solution. Radioactivity measurements were made by scintillation counting. Cumulative [^{14}C]carbon dioxide evolution was expressed as a percentage of the initially added radioactivity.

2.4 Second experiment

This compared the effects on atrazine mineralisation of glucose, cellulose and other more 'natural' carbon sources like green manure (rye-grass), wheat straw or poplar sawdust, representing substrates with different cellulose-to-lignin ratios. We also tried to relate the observed modifications in the kinetics of atrazine mineralisation to changes in the size or composition of the soil microflora.

As a first part of this experiment, kinetics of mineralisation were studied after supplying the soil with each substrate (2·5 or 2 g C-substrate kg^{-1} soil on the basis of an estimated percentage of 40 or 50% of organic carbon). The C/N ratio was adjusted to 10 with ammonium sulfate. When necessary, correction was made for the natural N content of the substrates (Table 3). Only [*ethyl*-^{14}C]atrazine was used in that experiment (3·33 mg kg^{-1} soil and 6·67 kBq per sample). Organic treatment and chemical application were made simultaneously at the beginning of the experiment. All other experimental parameters (type and structure of the soil, number of replicates, etc) and conditions (water content, incubation temperature, design of the incubator etc.) were set as described for the first experiment. The cumulative evolution of [^{14}C]carbon dioxide was measured and expressed as a percentage of initially added radioactivity.

A second part of this experiment consisted in following the variations of size of the soil microbial biomass as a whole or by considering separately its bacterial and fungal components. Triplicate soil samples (20 g) were either supplied with the different organic amendments already described or with non-labelled atrazine (3·3 or 33·3 mg kg^{-1} soil) or left unamended. Microbial

biomass measurements by fumigation-incubation and plate counts of culturable bacteria and fungi were made at the beginning of the experiment using non-enriched soil and after 14, 28, 63 and 130 days in the amended soils. The fumigation-incubation procedure was applied as described elsewhere.[31] The total bacterial (plus actinomycetes) microflora was counted on solid YPGA medium (50 µl deposits per Petri dish of the 10^{-4}, 10^{-5} and 10^{-6} soil dilutions). Each dilution was repeated five times. Readings were made after incubation at 28°C for 48 h. The fungal microflora was determined on solid acidified malt extract medium by pouring the soil dilutions (1 ml of the 10^{-3}, 10^{-4} and 10^{-6} dilutions) into the culture medium (10 ml) before it was solidified. Ten replicates were prepared for each dilution and readings were made after six days of incubation at 20°C.

3 RESULTS AND DISCUSSION

Figure 1a–h summarises the results on the influence of glucose and cellulose on kinetics of mineralisation of

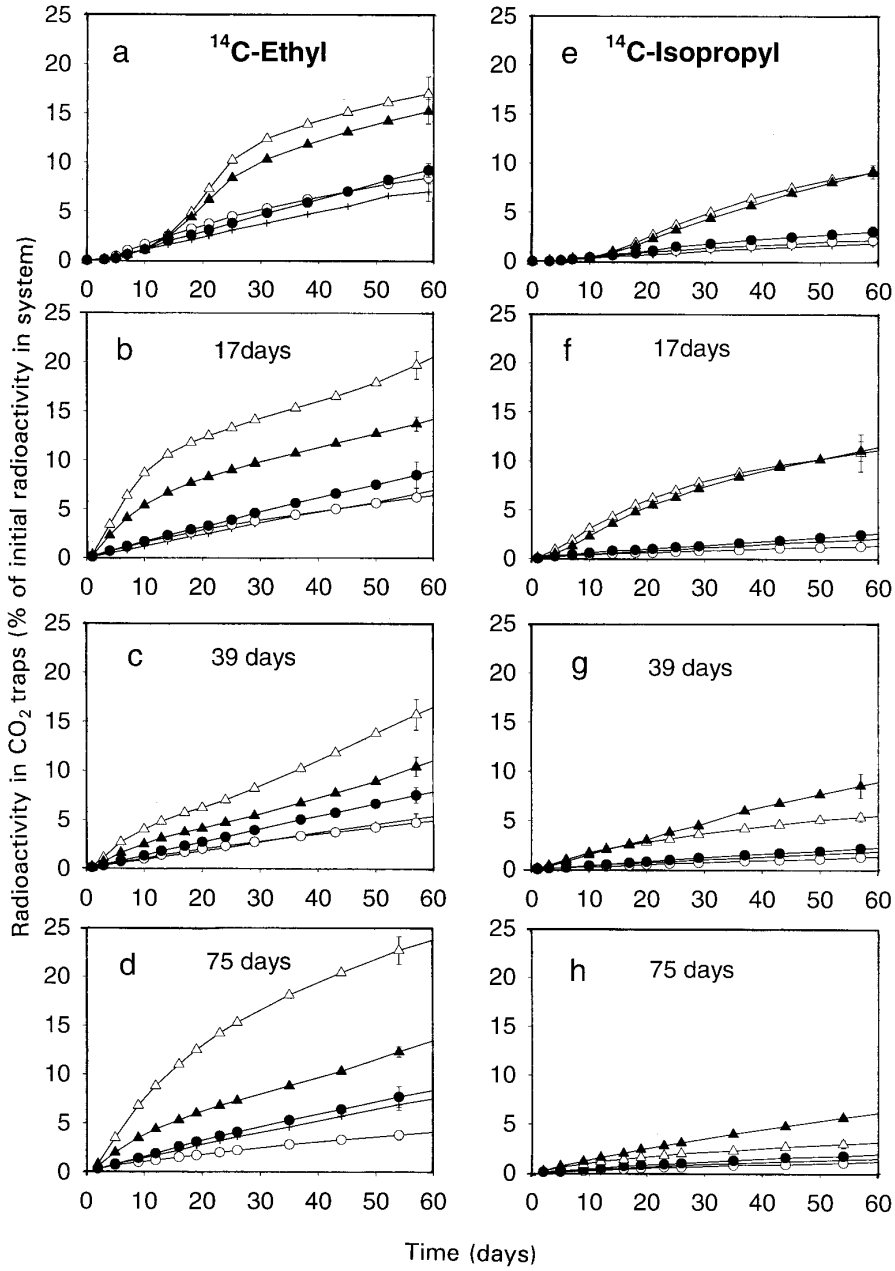

Fig. 1. Effects of cellulose (0·5% C/N 10: Δ; 0·5% C/N 50: ▲) and glucose (0·5% C/N 10: ○; 0·5% C/N 50: ●) amendments as compared to the control (+) on the kinetics of mineralisation of [*ethyl*-^{14}C (a,b,c,d) and [*isopropyl*-^{14}C] atrazine (e,f,g,h). Organic amendments have been supplied simultaneously (graphs a,e) or 17 days (graphs b,f), 39 days (graphs c,g) and 75 days (graphs d,h) prior to atrazine treatment.

both ethylamino and isopropylamino side-chains of atrazine. The effect of C/N ratios and different residence times of the organic substrates are shown. The first observation is that the soil has a very low natural capacity to mineralise atrazine side-chain C. After two months of incubation with the pesticide, the cumulative percentages of mineralisation never exceed 10% of the initial radioactivity for the ethylamino side-chain and 3% for the isopropylamino side-chain. This result has been confirmed recently.[32] With glucose, no significant increase in side-chain C mineralisation activity is observable when both the organic substrate and the pesticide are added simultaneously. Delayed pesticide application results, at best, in a cancellation of this effect in the case of a limited inorganic N supply or even in its reversal when nitrogen does not limit utilisation of supplemental C. The situation is quite different with cellulose which, in all circumstances, significantly stimulates atrazine side-chain mineralisation. It is well known that application of inorganic nitrogen enhances cellulose breakdown in soil and that the rate of decomposition is proportional to the concentration of nitrogen up to a certain level after which cellulose decomposition does not respond to supplemental increments. The point at which additional quantities would be no longer beneficial is at a ratio of 1 part of inorganic nitrogen to 35 parts of cellulose.[33] In our study, ethylamino side-chain mineralisation is best stimulated for the lower value of the C/N ratio (10) with cumulative percentages of evolved radioactivity after 60 days varying between 16 and 23% for different periods of preliminary incubation with the organic amendment. The pre-incubation results in an immediate start of atrazine mineralisation and progressively increases the advantage offered by an excess in supplemental N (C/N 10) as compared to a deficit (C/N 50). Interestingly, prior incubation with cellulose for 17 days (Fig. 1b) and 39 days (Fig. 1c) induces multiphasic mineralisation kinetics with some perceptible increase in the rate of [^{14}C]carbon dioxide evolution 35 days and 20 days, respectively, after herbicide application. By comparison, for the isopropylamino side-chain, the influence of the C/N ratio of the organic amendment is of no significance if application of the pesticide follows organic treatment within two- to three-week period, after which a small deficit in N supply has a definite beneficial effect on mineralisation. Two-month kinetics of mineralisation resulted in recoveries of radioactivity evolved as [^{14}C]carbon dioxide in a range between 7 and 12% of that initially added. It is also worth mentioning that when cellulose is the organic substrate preliminary incubation of the organic amendment before chemical application results in the disappearance of the 15-day lag phase preceding side-chain mineralisation.

It has been argued that atrazine can serve as an alternative N source especially after addition of easily degradable carbon substrates.[10] Due to the presence of a large inorganic N in our experiment, the pressure to use N derived from atrazine is not extreme.

The results of the second experiment are shown in Fig. 2 for mineralisation kinetics of atrazine in the presence of different organic material and Fig. 3a–c for time-course biomass measurements and bacterial and fungal communities countings. It is again demonstrated that addition of organic material has significant beneficial consequences on mineralisation of the ethylamino side-chain C of atrazine. Cellulose is the most efficient carbon source, with 28% atrazine mineralised after two months, but other more 'natural' substrates, essentially plant material such as straw, sawdust or green manure, in that order, have been proved to stimulate dealkylation of atrazine as well. They are respectively associated with 24, 23 and 19% mineralisation, as compared to only 10 and 11% in the control and glucose-amended soils. The lag phase that was evident in the first experiment under similar conditions is reduced to less than five days. In order to derive a causal relationship between decay of plant tissues and enhanced atrazine mineralisation, the microbial biomass has been measured and the bacterial and fungal counts determined. After normalisation with respect to the increase in microbial C in the unamended control, i.e. 278 mg kg^{-1}, differential microbial C increments in the treated soils correspond to assimilation yields of 31·1, 21·9, 18·8, 17·5 and 15·7% for glucose, rye-grass, cellulose, straw and sawdust respectively; the higher lignin content might be responsible for the relative resistance of straw and sawdust. The maximal microbial biomass is obtained after two weeks for glucose and atrazine at the highest concentration and only four weeks for the control and all other amended soils. There is a poor correlation between the rankings of the organic substrates according to their ability to be transformed into microbial C or to their potential to stimulate atrazine

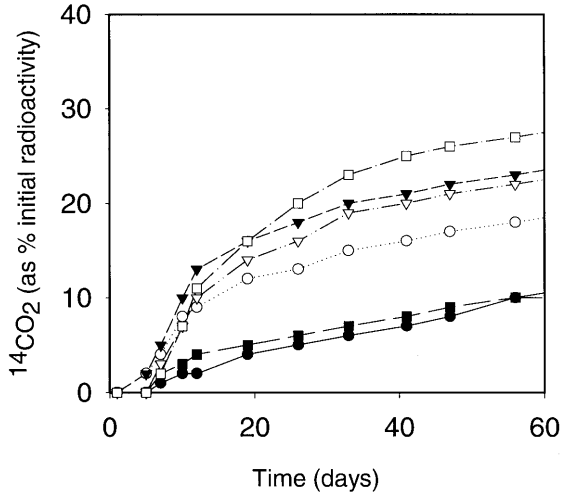

Fig. 2. Effects of (○) grass, (▼) straw, (▽) sawdust, (■) glucose, and (□) cellulose amendments as compared to the control (●) on the kinetics of mineralisation of [*ethyl*-^{14}C] atrazine.

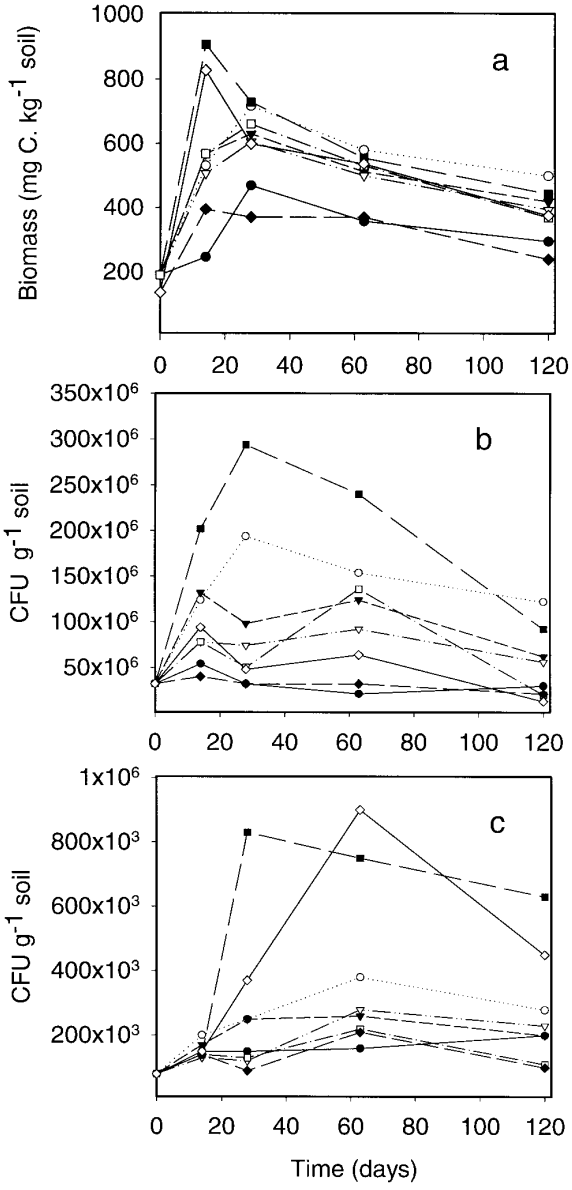

Fig. 3. (a) Effects of (○) grass, (▼) straw, (▽) sawdust, (■) glucose, and (□) cellulose amendments as compared to the control (●) on the total C.F.İ. microbial biomass and (b) on its bacterial and (c) fungal counts after applications of (◆) 3·2 mg kg^{-1} and (◇) 32 mg kg^{-1} atrazine

side-chain C mineralisation. This indicates that, although the size of the microbial biomass may be a convenient tool for predicting the rate of decomposition of xenobiotic compounds such as atrazine,[34] it is not the sole determinant. Counting experiments corroborate these main conclusions but a more detailed examination of the results reveals some distinct features. In all circumstances maximum counts are obtained only after four weeks. When glucose is used, an experimental artefact could explain the differential rate of development of the soil microflora. It has been demonstrated that the fumigation-incubation technique could be invalidated in the presence of substrates which are readily metabolised and can interfere with microbial carbon released after fumigation. Residual amounts of glucose or of intermediate compounds that can support microbial growth could account for the misleading observation of a rapid and important stimulation of the soil microflora after addition of glucose amendments. For all other substrates but cellulose, bacterial counts follow the same time-course variations in the same order of efficiency in C assimilation as those observed for the whole microbial biomass. Cellulose and, to a lesser extent, straw seem to provoke a more complex response of the soil microflora, with a first rise in number in the primary bacterial community feeding on the added and naturally occurring organic substrates. After falling to a level close to the cell count found in the unamended control, a second rise in bacterial number follows, indicating the late development of a secondary community growing on a residual amount of added C, waste metabolites and microbial protoplasm released from dead cells. The fungal microflora is also stimulated by addition of glucose and atrazine at the highest dosage, but is relatively unaffected by all other types of carbon substrate. These results are relatively unexpected if we consider that the more resistant carbonaceous components of plant residues are known to support preferentially a fungal microflora, and that even the higher dosage of atrazine does not provide enough C to sustain the observed fungal growth. Thus, it is not easy to determine the precise causal relationship of the enhanced degradation of atrazine in the presence of more readily degradable carbon and energy sources. Different factors such as the chemical composition and structure of the organic material, the C/N ratio of the amendment and its degree of maturation influence the composition of the soil microflora.

For simple organic compounds such as glucose and cellulose, the chemical structure is probably the main factor which determines the composition of the soil microflora. It is well known that addition to soils of simple sugars or succulent plant material prompts a rapid and preferential rise in the number of bacteria. On the other hand, more resistant carbonaceous compounds seem to support a microflora which is largely fungal. It has frequently been observed that, following treatment of soil with cellulose, there is a significant increase in the number of fungi, particularly if the nitrogen supply is adequate. For that reason, it has been argued that fungi are the main agents of cellulose degradation, particularly in humid and acid habitats, as opposed to bacteria that could be the causative organisms in dry and neutral environments.[33] Yet, in the present investigation, there is no evidence of a significant rise in abundance of the fungal community to give support to the assumption that the cellulolytic fungal microflora could be chiefly responsible for atrazine dealkylation. Examination of estimates of the soil microbial biomass and counting of the bacterial microflora may offer an alternative explanation. It is evident

that biomass and bacterial density are influenced to a large extent by cellulose, with bacterial numbers varying according to the successive proliferation of two communities. The lag period that precedes enhancement of atrazine dealkylation when both cellulose and atrazine are applied simultaneously could signify that more numerous and/or active degraders can be found among bacteria which make up the secondary flora. The initial step of cellulose conversion into its oligo- and monomeric building blocks by the primary cellulolytic flora may also provide other non-cellulolytic microbial species, including atrazine degraders, with enough carbon and energy sources making up a pool of available co-substrates to initiate the co-metabolic transformation of the atrazine side-chain C. It is also possible that the amount of available oligo- or monomeric sugars plays a role in the duration of the lag phase. From preliminary experiments (Soulas, G. and Fondeur, J. M., unpublished results) it has been observed that repeated additions of minute amounts of glucose stimulate mineralisation of atrazine to a larger extent than a single addition equivalent to the total amount. This observation could be fully understood if it is suggested that the atrazine dealkylation enzyme system is inducible and subjected to catabolite repression by glucose, a physiological trait which has been described for many other inducible enzyme systems and which may result in diauxic growth. In the present study, degradation of atrazine would start only after the amount of glucose had been reduced to a level which is compatible with induction of the atrazine dealkylation enzyme system.

As with 2,4-D,[35] we have observed a late recovery in ethylamino side-chain mineralisation activity which could be in agreement with the assumption of the late development of a microbial, possibly fungal, community living at the expense of more recalcitrant humic structures after labile C substrates have been exhausted. The beneficial effect resulting from the addition of organic residues with a high lignin content such as straw or sawdust does not contradict this assumption. The apparent stimulation in the production of microbial biomass after two weeks and of fungi after two months has no evident explanation if we consider that the total amount of C from the pesticide, 14·6 mg kg^{-1} soil, is far from being sufficient to explain observed microbial growth. Other biological effects such as, for instance, dormancy repression might be involved.

4 CONCLUSION

Working with a soil showing a naturally low capacity to mineralise atrazine, it has been demonstrated that it is possible to substantially stimulate side-chain mineralisation of this herbicide by enriching the soil with C sources such as cellulose, green manure, straw or sawdust but not with glucose. It has been shown that the chemical nature of the C source is the predominating factor, with cellulose being the most efficient substrate in accelerating atrazine side-chain mineralisation. Attempts to modify atrazine degradation can also be achieved by adjusting the C/N ratio of the organic amendment, which has been proved to play a significant role in the mineralisation of the ethylamino side-chain but not in the mineralisation of the isopropylamino side-chain. Accelerated mineralisation of atrazine seems to result from activation of microbial species able to co-metabolise atrazine side-chain C after easily available and readily metabolisable low-molecular-weight co-substrates have been released from polymerised C substrates. Glucose produced by depolymerisation of cellulose might induce catabolic repression of dealkylation enzyme systems and be responsible for a lag phase in atrazine side-chain mineralisation.

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